Kidney International, Vol. 49 (1996) pp. 153–157

Receptors in proximal tubular epithelial cells for tubulointerstitial nephritis antigen

YONG CHEN, UMA KRISHNAMURTI, ELIZABETH A. WAYNER, ALFRED F. MICHAEL, and ARISTIDIS S. CHARONIS

Departments of Laboratory Medicines and Pathology and Pediatrics, University of Minnesota Medical School, Minneapolis, Minnesota, USA

Receptors in proximal tubular epithelial cells for tubulointerstitial nephritis antigen. Tubulointerstitial nephritis antigen (TIN-ag) is a novel basement membrane macromolecule that is involved in human anti-tubular-basement-membrane-mediated tubulointerstitial nephritis. The presence of antibodies to TIN-ag may result in an alteration of proximal tubule epithelial cell interaction with surrounding matrix and contribute to the pathogenesis of immunc-mediated tubulointerstitial disease. To study the adhesive interactions between TIN-ag and proximal tubule epithelial cells and the macromolecules that mediate these interactions, an immortalized proximal tubular epithelial cell line from normal adult human kidney (HK-2) was used. Plastic-coated TIN-ag was able to promotc adhesion of HK-2 cells in a concentration-dependent manner. The strength of the adhesive interaction was comparable to that of type IV collagen or laminin. To explore which members of the integrin family of cell surface receptors were involved in this interaction, we performed fluorescence activated cell sorting (FACS) analysis and adhesion-inhibition studies using monoclonal antibodies against various integrins. Both approaches suggested that integrins $\alpha 3\beta 1$ and $\alpha v\beta 3$ are crucial for the adhesion of proximal tubule epithelial cells on TIN-ag, and that they are probably using independent domains of TIN-ag for their action. These data will help us to understand the interactions between proximal tubule epithelial cells and the underlying basement membrane, and will provide clues to the pathogenesis of kidney tubular diseases at the molecular level.

TIN-ag has been described as a 58 kD basement membrane macromolecule recognized by human autoantibodies in certain forms of tubulointerstitial nephritis [1]. It is a basement membrane glycoprotein with restricted distribution: it mainly exists in the renal cortex and to a lesser extent in the ileum, and in much smaller amounts in the corneal and the epidermal basement membrane [2]. The unique pattern of distribution of TIN-ag suggests that it may contribute to a specific structural conformation of these basement membranes, possibly related to specialized functions of the overlying cells. This notion has been reinforced by *in vitro* studies that have demonstrated that isolated TIN-ag interacts in a specific way with type IV collagen and laminin, and has the ability to interfere with laminin polymerization or pre-existing laminin polymers [3]. Basement membrane macromolecules also interact with cell surface components, mediating adhesive events which provide cells with anchorage, traction for migration and signals controlling various cellular functions [4]. The main family of cell surface receptors mediating adhesive events is the family of integrins. Integrins are heterodimeric transmembrane glycoproteins consisting of an α-chain and a β-chain [5]. The activities of many integrins can be modulated by cells and they, in turn, can modulate cellular activities in ways that extend far beyond adhesion [6, 7]. TIN-ag is an important component of renal tubular basement membrane, and its interactions with proximal tubule epithelial cells might be crucial for certain aspects of cellular function. In the present study, we examined the role of several integrins in mediating adhesion to TIN-ag, using a cell line of human tubular epithelial cells.

Received for publication May 19, 1995
and in revised form August 15, 1995
Accepted for publication August 18, 1995

Methods

Cell lines and culture conditions

HK-2 was characterized as described previously [8]. The cells were cultured in keratinocyte serum-free medium (K-SFM, Gibco Life Technologies, Grand Island, NY, USA), supplemented with epidermal growth factor (EGF; 10 ng/ml) and bovine pituitary extract (40 μg/ml). The cells were grown in T-75 flasks until 75 to 80% confluent and labeled for 18 hours with 0.5 mCi of (^{35}S)-methionine (Du Point/NEN, Boston, MA, USA) per T-75 flask.

Monoclonal antibodies to integrin

Monoclonal antibodies (mAbs) to the integrins $\alpha 2$(P1H5), $\alpha 3$(P1B5), $\alpha 4$(P4G9), $\alpha 5$(P1D6), αv(P3G8), $\alpha v\beta 5$(P6H9), and $\beta 1$(P5D2) are previously described [9–12]. Dr. Martin Hemler (Dana-Farber Cancer Institute, Boston, MA, USA) supplied the antibody against $\alpha 1$ (TS2/7). Antibodies against $\alpha 6$(G0H3), $\alpha v\beta 3$ (Lm609) and $\beta 3$ were purchased from AMAC Inc. (Westbrook, ME, USA) and Chemicon International Inc. (Temecula, CA, USA), respectively.

Preparation of adhesive proteins

TIN-ag was extracted and purified from rabbit kidney cortex basement membrane by collagenase digestion and ion-exchange and gel permeation chromatography as previously described [1]. Briefly, to isolate basement membrane, frozen rabbit kidney cortices (Pel-Freez Biologicals, Rogers, AR, USA) were thawed by immersion in proteinase inhibitor buffer. Cortices were homogenized with a Polytron tissue disrupter and then passed through a 35-mesh sieve. The filtrate was concentrated by centrifugation (2800 × g, 10 min), resuspended, sonicated, and washed by

repeated centrifugation and resuspension. The basement membrane prepared with this procedure was incubated overnight at 37°C with 1 mg of Collagenase (from clostridium histolyticum, code: CLSPA, Worthington Biochemical Corp.) per 10 ml of wet tissue. The sample was subjected to cation exchange chromatography on a 25 ml S-Sepharose column, then further purified by a Sephacryl S-300 gel filtration column. The fractions containing TIN-ag were pooled and concentrated. All TIN-ag isolation steps were done at 4°C. The purified TIN-ag was stored at −80°C. Laminin and type IV collagen were extracted from EHS tumor grown subcutaneously in lathyritic mice according to protocols previously described [13, 14]. Laminin was stored in liquid nitrogen and type IV collagen was stored on ice. Before use, all macromolecules were cleared of large aggregates by centrifugation.

Fluorescence activated cell sorting (FACS)

Expression of integrin subunits on cultured HK-2 was evaluated by indirect immunofluorescence staining and FACS. HK-2 were released with trypsin, washed and resuspended in FACS buffer (HBSS, 2% goat serum, 0.02% sodium azide). An equal number of cells, 2×10^5 were added to each vial. The cells were incubated with monoclonal antibodies to $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\beta 1$, $\beta 3$, αv, $\alpha v \beta 3$, $\alpha v \beta 5$, HLA (used as a positive control), and normal mouse immunoglobulin G (used as a negative control) for one hour at 4°C and washed once with 1 ml FACS buffer. Anti-mouse immunoglobulin G conjugated to fluorescein isothiocyanate (FITC) was then added in a total volume of 0.5 ml FACS buffer and incubated for 30 minutes at 4°C. The cell were again washed in 1 ml of FACS buffer and resuspended in 0.5 ml of 1.5% formaldehyde. The data were analyzed using CONSORT 30 software on a FACScan (Becton Dickinson, Mountain View, CA, USA). Positive fluorescence was determined on a four decade log scale and fluorescence (log F1) was expressed as the number of percentage immunoflurescence positive cells.

Cell adhesion to TIN-ag, type IV collagen, and laminin

Before metabolically labeling HK-2 with (^{35}S)-methionine, HK-2 medium was changed to medium lacking bovine pituitary extract. Cells at 70% confluency were rinsed twice with Hank's balanced salt solution (HBSS) and detached from the flask with trypsin (0.05%)/EDTA. Ninety-six well plates were coated overnight at 29°C with 50 μl of TIN-ag, type IV collagen, and laminin in serial dilution starting from 200 μg/ml (10 μg/well). Under these conditions 32% TIN-ag, 43% type IV collagen, and 40% laminin adhered [3, 15]. To block the remaining reactive sites the plates were treated with 200 μl of BSA at 2 mg/ml for two hours at 37°C. A total of 50 μl of suspension containing 6000 cells were added per well and the plates were incubated at 37°C in a humidified incubator for 60 minutes. The cells were then washed three times with the medium to remove nonadherent cells and 100 μl of "lysis" buffer (0.5 NaOH, 1% SDS in distilled water) was added to each well for 50 minutes at 60°C. The lysate was transferred to scintillation vials and counted. The data were expressed as a percentage of the total input cpm. Cell adhesion assays were performed in quadruplicate.

For competition experiments 96 well plates were coated with 50 μl of TIN-ag at 10 μg/ml (0.16 μg/well), overnight at 29°C. The plates were BSA-"blocked" for two hours and then serial dilutions of hybridoma culture supernatant containing known quantities of antibody was added to each well, in quadruplicate, starting at more than 10 μg/ml and 100 μl/well. Cells were processed as described above and were added to each well in 50 μl (6000/well) at 37°C for 60 minutes. Then non-adherent cells were washed off and bound cells were quantitated as previously described. Data were expressed as the percent of maximal binding observed in the absence of antibody. Inhibition experiments were performed in quadruplicate.

All experiments described above were performed at least twice.

Results

Adhesion experiments

To examine the role of TIN-ag in the adhesion of HK-2 cells, increasing concentrations of TIN-ag, type IV collagen, laminin (both used as positive controls) and bovine serum albumin (BSA) (used as a negative control) were absorbed in 96-well plastic plates. Metabolically labeled HK-2 cells were treated as described in the **Methods** section and then added to the wells. At the end of the incubation period (60 min) adherent cells were lysed and the lysate was counted. The data shown in Figure 1A suggest that TIN-ag is a strongly adhesive protein, at least for the HK-2 cells. The same data, expressed per molar concentration of absorbed protein retained on the plastic well, are shown in Figure 1B. Again it is apparent that for this cell line TIN-ag mediates adhesive events in a concentration-dependent fashion. The data of Figure 1B also suggest that for HK-2 cells TIN-ag acts as a stronger adhesion molecule compared to laminin, but that it is by an order of magnitude weaker than type IV collagen in its adhesive ability.

Presence of integrins on the cell surface of HK-2 cells

It is well accepted that integrins are the major class of cell surface macromolecules mediating cellular adhesion to extracellular matrix components. Therefore, we examined the presence of several integrin subunits on the cell surface of HK-2 cells using the FACS technique. Monoclonal anti-integrin antibodies were used as primary antibodies. As secondary antibodies we used anti-mouse antibodies coupled to FITC. As negative control, nonspecific mouse immunoglobulin was utilized, and under these conditions, only 5% of the cells were positive. As a positive control, we have used an anti-HLA monoclonal antibody; in this case, more than 65% of the cells were positive. The data shown in the histogram of Figure 2 suggest that the integrin subunits $\alpha 3$, αv, and $\beta 1$ and the complex $\alpha v \beta 3$ were expressed whereas the subunits $\alpha 1$, $\alpha 2$, $\alpha 4$, $\alpha 5$, and $\alpha 6$ were not expressed or were minimally expressed on the surface of HK-2 cells. These results provided evidence that the main integrins found on HK-2 cells were probably $\alpha 3 \beta 1$ and $\alpha v \beta 3$, and indirectly suggested that these integrins should be primary mediators of cellular adhesion to TIN-ag.

Effects of anti-integrin antibodies on the adhesion of HK-2 cells on TIN-ag coated plates

To confirm the importance of specific integrins in the phenomenon of adhesion of HK-2 cells on TIN-ag, the following functional assay was used: in 96-well plastic plates coated with TIN-ag as described, increasing concentrations of monoclonal anti-integrin antibodies were added. In each well, the same number of cells was then added and after 60 minutes the extent of adhesion was quantitated as described. A number of monoclonal antibodies like

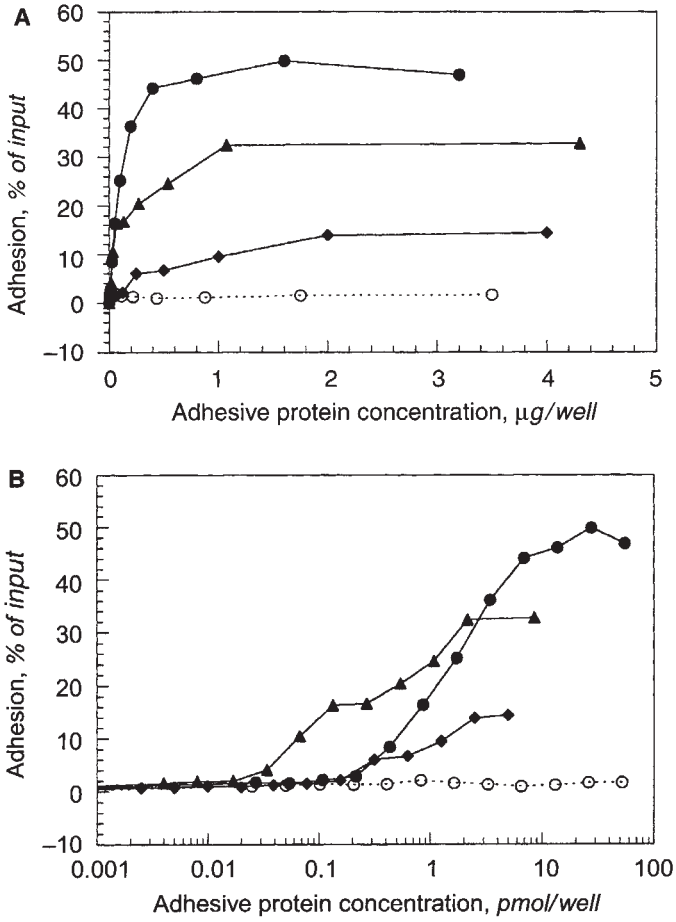

Fig. 1. *(A) HK-2 adhesion to basement membrane proteins, expressed as μg retained on plastic well.* Values are means of 2 to 3 experiments, performed in quadruplicate. (B) HK-2 adhesion to basement membrane proteins expressed as pmolar concentration retained on plastic well. Symbols are [●] TIN-ag; [◆] laminin; [▲] type IV collagen; [○] BSA.

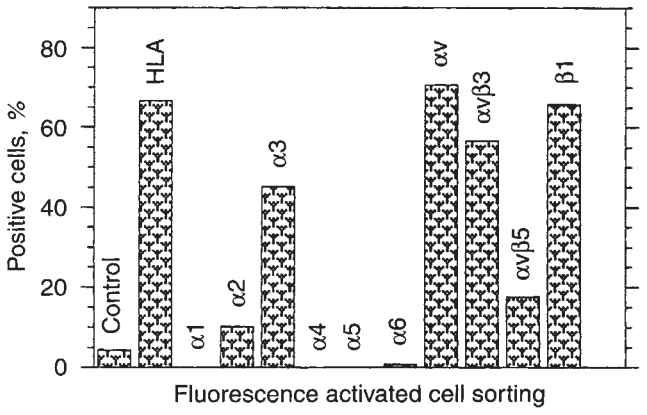

Fig. 2. *Distribution of integrin receptors on HK-2 cell surface, as determined by anti-integrin antibodies with FACS.*

anti-HLA (used as a control), anti-α1, anti-α4, anti-α5, and anti-α6 did not have any effect on the adhesion of HK-2 cells on TIN-ag (Fig. 3A). On the contrary, increasing concentrations of anti-α3, anti-αv, and anti-β1 resulted in inhibition of adhesion ranging from 80% to 50% (Fig. 3B). More specifically, anti-β1 antibody resulted in inhibition of adhesion by 80%, anti-α3 antibody achieved a 65% inhibition, and anti-αv antibody reached a 50% inhibition. Antibodies against αvβ3 achieved a 65% inhibition and antibodies against αvβ5 showed a 15% inhibition (data not shown). Interestingly, antibodies against α2 also showed a small (20%) inhibition (Fig. 3B).

In view of these results, it was of interest to test whether a combination of these antibodies would have an additive effect, producing higher levels of inhibition than the ones observed by using individual antibodies. The results of these experiments are shown in Figure 3C. It is obvious that with most combinations used, a nearly 100% inhibition of adhesion was achieved. The results shown in the three panels of Figure 3 strongly suggest that the main integrins mediating adhesion of HK-2 cells on plastic-coated TIN-ag are α3β1 and αvβ3.

Discussion

In this report, we provide evidence that TIN-ag can act as an adhesive basement membrane macromolecule for proximal tubule epithelial cells, and that this interaction is primarily mediated via integrins α3β1 and αvβ3. In our studies we have used a recently described cell line (HK-2), derived from normal adult human renal cortex proximal tubule epithelial cells exposed to a human papilloma virus (HPV 16), containing the E6/E7 genes. This cell line appears to be well differentiated on the basis of its morphological, enzymatic, cytochemical, and functional characteristics [8]. Therefore, it seemed an optimal choice for our studies, in view of the fact that the proximal tubule epithelial basement membrane is the site of the highest expression of TIN-ag. Studies performed with this cell line clearly establish TIN-ag as a major macromolecule that can mediate adhesion of proximal tubule epithelial cells. In comparison with laminin and type IV collagen, TIN-ag was found to be the most adhesive per mass of protein. Expression of the results per moles of protein indicated that TIN-ag is clearly more adhesive than laminin, but less adhesive than type IV collagen at low coating concentrations. At higher coating concentrations (above 1 pmol/well) TIN-ag was more adhesive than type IV collagen as well. Adhesion assays performed in the past using a transformed murine tubular epithelial cell line [3] gave slightly different results. These studies have hinted at the importance of TIN-ag as an adhesive macromolecule, but it was found to be per molar ratio, one to two orders of magnitude less adhesive than type IV collagen or laminin. Species differences may account for the slightly different results. It should also be kept in mind that all these experiments were performed with TIN-ag extracted from rabbit tissue; use of TIN-ag extracted from the same species will provide more conclusive results in the future.

Having established that TIN-ag can mediate adhesion of proximal tubule epithelial cells, it was important to examine which cell surface macromolecules are involved in this phenomenon. We decided to focus on the family of integrins, which are well recognized as the main mediators of cell-matrix interactions. This approach does not rule out other types of macromolecules as possible mediators of cellular adhesion to TIN-ag. However, the results obtained and the level of inhibition achieved strongly suggest that integrins must be by far the most important mediators

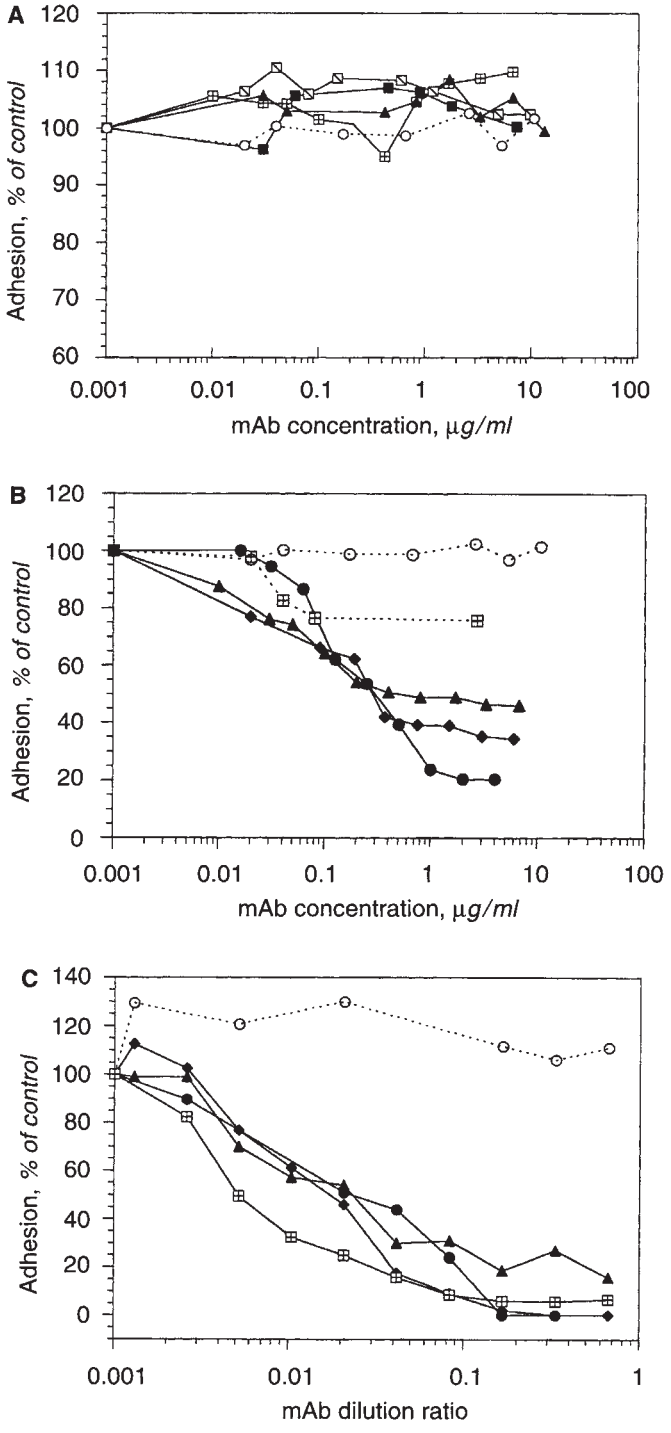

Fig. 3. *(A) mAbs inhibition of HK-2 adhesion to TIN-ag (0.16 μg/well) in a solid phase assay: [○] anti-HLA; [⊞] anti-α1, TS2/7; [■] anti-α4, P4G9; [⊠] anti-α5, P1D6; [▲] anti-α6.* (**B**) mAbs inhibition of HK-2 adhesion to TIN-ag (0.16 μg/well) in a solid phase assay. Symbols are: [○] anti-HLA; [⊞] anti-α2, P1H5; [◆] anti-α3, P1B5; [●] anti-β1, P5D2; [▲] anti-αv, P3G8. (**C**) Combined mAbs inhibition of HK-2 adhesion to TIN-ag (0.16 μg/well) in a solid phase assay. Symbols are: [○] anti-HLA; [●] anti-β1+anti-αv; [◆] anti-β1+anti-αvβ3; [▲] anti-αv+anti-α3; [⊞] anti-α3+anti-αvβ3.

of cell adhesion to TIN-ag, at least in the case of proximal tubule epithelial cells. The obtained results from FACS correlated well with the inhibition studies and strongly suggested the involvement of integrins α3β1 and αvβ3. These two types on integrins are reported to be present *in situ* in the kidney proximal tubule [16], although this finding is not substantiated in other reports [17, 18]. Additionally, high levels of expression of these integrins have been observed in a primate-derived kidney epithelial cell line [19]. In view of the data presented, the current belief that in the proximal tubule α3β1 integrin is used for attachment to laminin and αvβ3 for attachment to collagen [20] should be revised to accommodate TIN-ag as an additional ligand for these two integrins. The monoclonal antibodies used achieved a 50 to 80% inhibition individually. This level suggested that the adhesive interactions should be the result of at least two independent integrins, acting simultaneously. Confirmation of this hypothesis came from the experiments where monoclonal antibodies were combined; in these experiments, 100% inhibition was achieved. It should be kept in mind that proximal tubule epithelial cells *in vivo* might express more integrins on their surface than the cell line tested in the present study, and that some of these other integrins may also contribute to or modify the adhesion to TIN-ag. Furthermore, other cell types interacting with TIN-ag may use different integrins for adhesion.

Cells often display multiple integrins capable of interacting with a particular extracellular matrix protein [5]. In most of these instances, the integrin receptors interact with different regions of the ligand macromolecule [6, 7]. Distinct integrins that recognize the same ligand may convey different signals intracellularly [6, 7]. Therefore, it would be of great interest in the future to precisely map the sites of interaction with the integrins α3β1 and αvβ3 on the amino acid sequence of the TIN-ag.

Understanding the molecular mechanisms operating in the attachment of kidney tubular epithelial cells to the underlying basement membrane is of major importance. In situations like acute renal failure, tubular epithelial cells become detached; this phenomenon is not resulting from cell death, because these cells have been shown to be up to 100% viable after their detachment [20]. It is remarkable that the majority of detached cells is from the proximal tubule section. In this section, TIN-ag is found in large quantities in the basement membrane. Therefore, defects in cell adhesion to this macromolecule might be involved in the pathogenesis of the detachment process. However, at present experimental evidence to support this hypothesis is lacking.

Acknowledgments

This work was supported by NIH grant DK-36007 (to ASC), by an American Heart Association grant (to ASC) and by NIH grant AI-0704 (to AFM). We thank Dr. Richard Zager for providing the line of HK-2 cells, Mr. Howard Higson for culturing the HK-2 cells and Drs. Theodosia Kalfa and Suman Setty for important suggestions during the course of this work.

Reprint requests to Aristidis S. Charonis, M.D., Ph.D., Box 609 UMHC, Department of Laboratory Medicine and Pathology, University of Minnesota Medical School, Minneapolis, Minnesota, USA

References

1. Butkowski RJ, Langeveld JPM, Wieslander J, Brentjens JR, Andres GA: Characterization of a tubular basement membrane

component reactive with autoantibodies associated with tubulointerstitial nephritis. *J Biol Chem* 265:21091–21098, 1990
2. BUTKOWSKI RJ, KLEPPEL MM, KATZ A, MICHAEL AF, FISH AJ: Distribution of tubulointerstitial nephritis antigen and evidence of multiple forms. *Kidney Int* 40:838–846, 1991
3. KALFA TA, THULL JD, BUTKOWSKI RJ, CHARONIS AS: Tubulointerstitial nephritis antigen interacts with laminin and type IV collagen and promotes cell adhesion. *J Biol Chem* 269:1654–1659, 1994
4. PAULSSON M: Basement membrane proteins: Structure, assembly, and cellular interactions. *Crit Rev Biochem Mol Biol* 27:93–127,1994
5. RUOSLAHTI E: Integrins. *J Clin Invest* 87:1–5, 1991
6. HYNES RO: Integrins: Versatility, modulation and signaling in cell adhesion. *Cell* 69:11–25, 1991
7. JULIANO RL, HASKILL S: Signal transduction from the extracellular matrix. *J Cell Biol* 120:577–585, 1993
8. RYAN MJ, JOHNSON G, KIRK J, FUERSTENVERG SM, ZAGER RA, TOROK-STORB B: HK-2: An immortalized proximal tubule epithelial cell line from normal adult human kidney. *Kidney Int* 45:48–57, 1994
9. WAYNER EA, GIL SG, MURPHY F, WILKE MS, CARTER WG: Epiligrin, a component of epithelial basement membranes, is an adhesive ligand for $\alpha 3\beta 1$ positive lymphocytes. *J Cell Biol* 121:1141–1151, 1993
10. WAYNER EA, CARTER WG: Identification of multiple cell adhesion receptors for collagen and fibronectin in human fibrosacoma cells possessing unique α and common β subunits. *J Cell Biol* 105:1873–1884, 1987
11. WAYNER EA, GARCIA-PARDO A, HUMPHRIES MJ, MCDONALD JA, CARTER WG: Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. *J Cell Biol* 109:1321–1330, 1989
12. WAYNER EA, ORLANDO RA, CHERESH DA: Integrins $\alpha v\beta 3$ and $\alpha v\beta 5$, contribute to cell attachment to vitronectin but differentially distribute on the cell surface. *J Cell Biol* 113:919–929, 1991
13. KLEINMAN HK, MCGARVEY ML, LIOTTA LA, GEHRON-ROBEY P, TRYGGVASON K, MARTIN G: Isolation and characterization of type IV procollagen, laminin and heparan sulfate proteoglycan from EHS sacroma. *Biochemistry* 21:6188–6193, 1982
14. CHARONIS AS, TSILIBARY EC, YURCHENCO PD, FURTHMAYR H: Binding of laminin to type IV collagen: A morphological study. *J Cell Biol* 100:1848–1853, 1985
15. HAITOGLOU CS, TSILIBARY EC, BROWNLEE M, CHARONIS AS: Altered cellular interaction between endothelial cells and nonenzymatically glycosylated laminin/type IV collagen. *J Biol Chem* 267:12404–12407, 1989
16. SIMON EE, MCDONALD JA: Extracellular matrix receptors in the kidney cortex. *Am J Physiol* 259:F783–F792, 1990
17. COSIO FG, SEDMAK DD, NAHMAN NJ: Cellular receptors for matrix proteins in normal human kidney and human mesangial cells. *Kidney Int* 38:886–895, 1990
18. KORHONEN M, YLANE J, LAITINEN L, VIRTANEN I: The alpha 1 - alpha 6 subunits of integrins are characteristically expressed in distinct segments of developing and adult human nephron. *J Cell Biol* 111: 1245–1254, 1990
19. GAILIT J, COLFLESH D, RABINER I, SIMONE J, GOLIGORSKY MS: Redistribution and dysfunction of integrins in cultured renal epithelial cells exposed to oxidative stress. *Am J Physiol* 264:F149–157, 1990
20. GOLIGORSKY MS, LIEBERTHAL W, RACUSEN L, SIMON EE: Integrin receptors in renal tubular epithelium: New insights into pathophysiology of acute renal failure. *Am J Physiol* 264:F1–F8, 1990